CULTIVATING CONDITIONS INFLUENCE INVERTASE PRODUCTION BY *ASPERGILLUS NIGER* IN SUBMERGED CULTURE

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ABSTRACT

Recently, there was increased in the production of microbial invertase due to its function which was important in production of invert sugar and high fructose syrup as compared to production of high fructose syrup and invert sugar for formulation of pharmaceutical product from sucrose by using acid hydrolysis. Therefore this study was conducted to examine the effect of substrate concentration by using sucrose from table sugar as cheaper carbon source of to provide an alternative production without implement other steps to secure the carbon source to be used in microbial invertase production by *Aspergillus niger*. This study was also conducted to study effect of pH and agitation speed on cultivating conditions by One Factor at a Time (OFAT) and eventually optimize the parameters for invertase production in submerged culture by using central composite design (CCD) in Design Expert for Response Surface Methodology (RSM) in submerged fermentation. The studied parameter were substrate concentration in selected range 10-50% (w/v), pH in range of pH 4.5-6.5, and agitation speed in range of 100-300 rpm. Studied conducted on one factor at a time yield maximum amount of invertase activity at 30 g/L sucrose concentration, pH 5.5 and 250 rpm with amount of 8.9132 IU/mL, 8.6754 IU/mL and 8.3429 IU/mL respectively after 40 hours fermentation period. After optimization of these three parameters using Response Surface Methodology (RSM), the optimum cultivating condition was obtained at 26.9583 IU/mL by using 30 g/L of sucrose concentration, pH 5.5 and 200 rpm. The optimum cultivating condition effect on invertase production in submerged culture was concluded at optimum condition obtain at 30 g/L sucrose concentration, pH 5.5 and 200 rpm from the study conducted by using central composite design since it had indicated that these three factors were significant with R^2 value of 0.9950 ($P<0.001$). Based on the result obtained from this study, it was recommended that table sugar can be used as carbon source and the model obtained can be further reviewed to ensure effective invertase production in submerged culture by *Aspergillus niger*.

ABSTRAK

Kebelakangan ini penghasilan invertase enzim daripada fermentasi oleh mikroorganisma semakin meningkat disebabkan oleh fungsi enzim ini yang dapat menghasilkan gula ringkas dan fruktosa berbanding penghasilan gula ringkas dengan menggunakan mekanisma penghasilan glukosa dan fruktosa daripada hidrolisis oleh asid. Oleh itu kajian dilaksanakan dengan meggunakan gula pasir sebagai substatu penghasilan invertase oleh *Aspergillus niger* dalam kultur kelalang bergoncang. Kajian juga dilaksanakan untuk mengkaji kesan pH dan kelajuan pengadukan terhadap penghasilan invertase dan seterusnya mengoptimumkan produktiviti dengan menggunakan rekabentuk komposit berpusat dalam perisian *Design Expert* untuk Kaedah Sambutan Permukaan oleh ferrmentasi di dalam media. Pembolehubah yang digunakan dalam kajian ini adalah 10-50(g/L) bagi kepekatan substratu, pH 4.5-6.5, dan kelajuan pengadukan dalam julat 100-300 rpm untuk kaedah satu faktor pada satu masa dan rekabentuk komposit berpusat. Keputusan yang optimum yang diperolehi untuk aktiviti invertase bagi kaedah satu factor pada satu masa adalah pada 30 g/L kepekatan sukrosa yang digunakan dalam medie, pH 5.5 and 250 rpm sebanyak 8.9132 IU/mL, 8.6754 IU/mL dan 8.3429 IU/mL bagi setiap faktor. Kaedah rekabentuk permukaan berpusat pula menunjukkan keadaan optimum yang boleh menghasilkan inverase adalah dengan menggunakan kepekatan sukrosa sebanyak 30 g/L pada pH 5.5 dan kejaluan pengadukan sebanyak 200 rpm kerana aktiviti invertase paling maksimum diperolehi dalam keadaan ini iaitu 26.9583 IU/mL dengan nilai R^2 iaitu 0.9950 ($P<0.001$). Konklusinya, keadaan optimum untuk menghasilkan invertase menggunakan kaedah fermentasi di dalam media ialah pada pH 5.5, 200 rpm dan kepekatan sukrosa sebanyak 30 g/L. Oleh itu disarankan kaedah fermentasi seperti ini yang menggunakan gula pasir dapat diguna pakai untuk menghasilkan enzim ini dengan membuat kajian lebih mendalam untuk penghasilan yang lebih efektif.

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X_3	Agitation speed of samples
B	Beta
X	Cell dry weight
b*ij*	Cross product coefficient
^0C	Degree Celcius
$	Dollar United States money
E	Error
Y	Invertase activity
$+\infty$	High factorial point
$-\infty$	Low level of factorial point
£	Model constant
%	Percentage
X_2	pH of samples
X_1	Sucrose concentration of samples

LIST OF ABBREVIATIONS

Adj R^2	Adjusted R^2
US $	United States Dollar unit
U/L.h	Units of enzyme per liter per hour
U/L	Units of enzyme per liter
U/g	Units of enzyme per gram
U	Unit of enzyme
Sp	Species
SHARP	Anomalous Patterson maps using subroutine XREP of program package SHELX 5.0 to refine the structure of invertase.
RSM	Response Surface Methodology
rpm	Revolution per minute
OFAT	One factor at a Time
NaOH	Sodium Hydroxides
NaCl	Sodium Chloride
mm	Millimeter
mM	Mill molar concentration
mL/min	Militre per minute
$MgSO_4.7H_2O$	Magnesium Sulphate Anhydrose
M	Molar unit concentration
LUNA NH_2 (AMINO)	Brand name of High Performance Liquid Chromatography Column.
KH_2PO_4	Potassium Dihyrogen Phosphate
HPLC	High Performance Liquid Chromatography
HCL	Hydrochloric Acid
GH32	Glycosides Hydrolyses 32 Family
FPLC	Fast Protein Liquid Chromatography
FOS	Fructooligosaccharides
$FeSO_4$	Iron (II) Sulphate
DOE	Design of Experiment
DMMULTI	Symmetry average initial phase in detection of solvent flattening invertase structure.
cm	Centimetre
CCD	Central Composite Design
ANOVA	Analysis of variance
Aa20	Strain number of *Aspergillus niger*
$(NH_4)_2 SO_4$	Ammonium Sulphate
(NH_4) CL	Ammonium Chloride
% w/v	Percentage of weight over volume

CHAPTER 1

INTRODUCTION

1.1 Introduction

Nowadays, there are high demand enzymes of food, drink, and confectionary industry. According to a fact from Enzymes for Food Processing Industry Project by Mott MacDonald, the author has reported that in the world enzyme market during year 2005 it is estimated about US $ 1000 million by the year which indicate that enzyme market offer high profit outcome and the market is continued growing .This increasing demand makes the requirement of enzymes production higher in industry. This requirement has developed much opportunity in industries regarding to market the potential way of production of enzyme with high profit income.

Invertase is a one of the beneficial enzyme that provides many products for industrial purpose such as pharmaceutical, food and etc. It is due to its utilization, its function to hydrolyze the sucrose into two equimolar mixtures of glucose and fructose at a concentration lower than 10 % sucrose (Guimaraes *et al.*, 2009). Therefore, this enzyme has attract researcher attention to study on any potential method of production that serves high effectiveness production method of microbial invertase although one previous method has been reported to produce this potential invertase in which the acid hydrolysis process but with low conversions efficiency which is 65-70% (Kaur and Sharma, 2005).

1.2 Problem Statement

Since microbial invertase production process is selected for this study, microorganism to be used in this study has become a serious matter to be observed. After some reviews, it is decided the *Aspergillus niger* is used to produce the invertase in submerged culture. However, there are many type of this species are discovered as the potential agent to produce this enzyme and therefore it is difficult to choose the most suitable type of *Aspergillus sp* for production process such as *Aspergillus niger* (Augur *et al*., 2000), *Aspergillus niveus* (Somera *et al*., 2009) and *Aspergillus fumigatus* (Gill *et al*., 2006). Therefore, the chosen type of *Aspergillus sp* must offer optimum production of invertase based on the effect of substrate concentration as well as the pH and the agitation speed on cultivating in submerged culture.

Problems arise from the substrate used in invertase production is one of the reason for this study to be conducted. It is because the local sucrose is expensive as compare to table sugar use as the substrate in this study. Therefore, new source of sucrose from table sugar in the market will be used to overcome this matter. The new selected sugar source should be able to produce higher invertase activity and cell biomass. Furthermore, since time is limitation factor to get the raw material, then this solution is used due to time saving method offered.

In fact, since the Response Surface Methodology is used in this study then time saving factor is become an important factor to be decided. Response Surface Methodology is more effective method to investigate the particular parameter that influences the invertase production in submerged culture compared to previous method that has been done by researcher before which is one-factor-at-a-time methods. This is because of the one-factor-at-a-time method is more time consuming and does not bring out the effect of interaction of various parameters (Elibol, 1999).

1.2 Research Objectives

The purpose of this study is to addresses an alternative method for production of invertase by selected microorganism and the utilization of cultivating condition for the growth of the selected microorganism.

The research objectives are:

1.2.1 To investigate the effect of substrate concentration (sucrose concentration), pH and the agitation speed on cultivating conditions for invertase production by *Aspergillus niger* in submerged culture.

1.2.2 To optimize the parameters for invertase production in submerged culture by using Response Surface Methodology (RSM).

1.3 Scope of study

This study is focusing on the scope as follows:

i. For the study of the effect of sucrose concentration influence the cultivating condition of invertase production in submerged culture by *Aspergillus niger*, a selected range of sucrose concentration is decided to be use from 10 until 50 % g/L.

ii. For the study on the effect of pH on the cultivating condition influencing the invertase production in submerged culture by *Aspergillus niger*, the range of pH use is pH 4.5, 5.0, 5.5, 6.0 and 6.5.

iii. For the effect of agitation speed that influence the cultivating condition of invertase production in submerged culture by *Aspergillus niger*, the range of rotation is selected in range 100 until 300 rpm.

iv. An optimization process of the parameters (sucrose concentration, pH and agitation speed) that influence the invertase production is conducted based on Response Surface Methodology (RSM) to achieve the most desirable condition of parameters for optimum level of invertase production.

1.4 Significance of Study

This study is believed to provide a new optimization method of invertase production by using microbial production method using *Aspergillus niger* with Random Surface Methodology (RSM) and provides a new alternative for raw materials of invertase production.

By using the Response Surface Methodology (RSM), a central composite design of experiment is used to investigate the optimum parameters (sucrose concentration, pH and agitation rate). This is because this method is more efficient compare to the using one-factor-at-a-time method that requires more times in conducting the experiment by shorting the time consuming for investigation of all parameters in this study.

Furthermore, a new potential source of raw material is evaluated for the purpose of producing lower cost invertase enzyme. Moreover, this raw material is easier than obtaining other carbon sources sucrose such using red carrot residue as substrate (Mona and Nooman, 2009) which require an extra method implemented in their production process to process the residue first before it is used as carbon source for invertase production. This will require more time to conduct the production of invertase enzyme and therefore it is wise to use local sugar as carbon sources that offer time saving method. Furthermore, this type of carbon source could be easily got in the market and cheaper than using commercial sucrose source from chemical company.

Moreover, this enzyme is widely used in confectionery industries and food industries. It is due to the characteristic that produce the fructose compound that have higher sweetening capacity, thus making this enzyme suitable for biotechnological applications, such as the production non-crystallizable sugars and soft centered chocolates (Rubio *et al.*,2003). It is also use to produce Fructooligosaccharides (FOS)

at higher concentrations of sucrose (Rustiguel *et al*., 2010). Furthermore, it has been used in the production of non-crystalling creams, jams, artificial honey and in confectionery industry (Emregul *et al*., 2007). Hence; study that related to production of this enzyme is encouraged.

In addition, this enzyme also can be used in pharmaceutical industry. It is useful for diabetics and potentiates iron absorption in children (Gill *et al*., 2006). Therefore, it is concluded that this study will be contribute large potential in market

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

A review is performed to identify studies that relevant to this study cultivating conditions influence invertase production by *Aspergillus niger*. The following keywords has been used to identify the relevant material for this topic; function of invertase, microorganism that use to produce invertase, production of invertase, previous parameter use to investigate invertase production and analysis of invertase. This research is basically about the findings of an effective way to increase the invertase production in submerged culture. Therefore this chapter provides five major topic reviews with its own subtopics on function of invertase, microorganism that use to produce invertase, production of invertase, previous parameter use to investigate invertase production and analysis of invertase.

2.2 The Invertase Enzyme

It is the one of the earliest enzyme discovered by researcher which is isolated in the second half of the 19^{th} century and it is become a valuable enzyme due to its own function which is to produced "invert sugar" in ratio of product mixture 1:1 of mixture of dextrorotatory D-glucose and levorotatory D-fructose (1) (Alberto *et. al*, 2004). Therefore, there is crucial to recognize the structure and function of invertase.

Alberto *et al.* have been proposed research paper that stated the structure of invertase are in folded structure as illustrated in the Figure 2.1 which was established in year 2004. Based on the Figure 2.1, it is in a, ribbon structure which represented of the

monomeric unit of *T. maritime* invertase with the highlighted N-terminal β-propeller module and the five blades (numbered *I–V*), and the C-terminal β-sandwich module (dark red) in section A and the *B*-section of the figure represent the experimental map after phasing with SHARP (25), solvent-flattening with DMMULTI (26), and non-crystallographic symmetry and averaging with RESOLVE (27) for the experimental electron density map, contoured at a 1σlevel, shows three antiparallel β--strands in the β-sandwich module at the C-terminal region of the protein. These two structures is the experimental data presented by Alberto *et al.* in his paper.

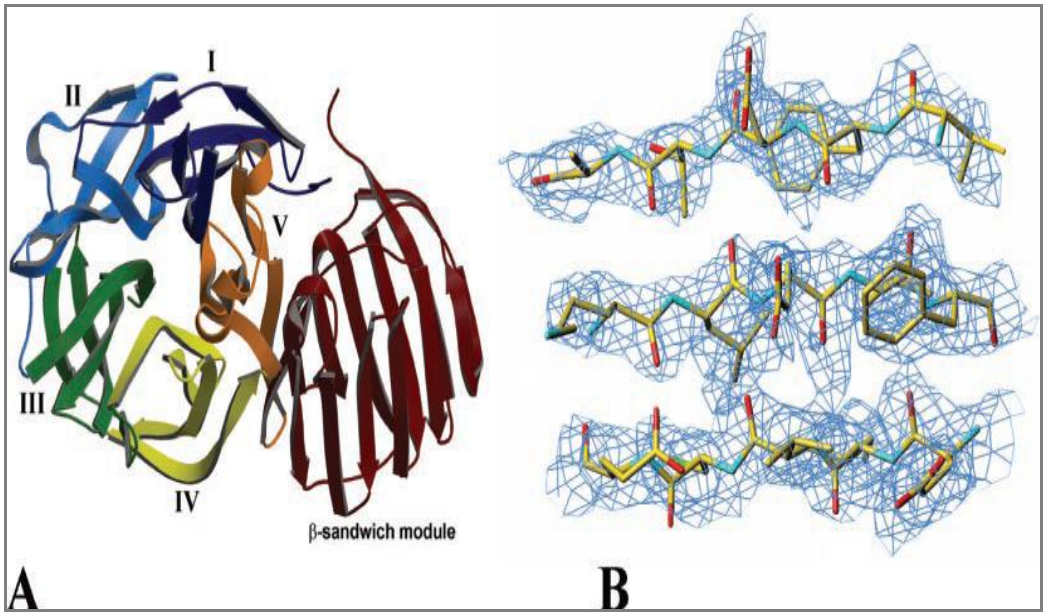

Figure. 2.1. The fold structure of invertase and experimental electron density map of invertase ribbon representation of the monomeric unit of *T. maritime.*

Source: Alberto *et al.*, (2004)

Since this type of enzyme is a high biotechnological potential β-D-fructofuranosidase, can be produced by many organisms, especially microorganisms like bacteria, yeast and filamentous fungi therefore it are produced in different formed by either intracellular or extracellular invertase (Rustiguel *et al.*, 2010).

This enzyme was not specifically defined in its structure until now. Researchers has proposed that a GH32 yeast invertase structure has not been reported until now, a

remarkable fact when taking into account that yeast invertase have been described as multimeric (Benito *et al.*,2010). Moreover, this research also proposed that the basic structural unit of intracellular and extracellular invertase is in dimer form as shown by electron microscopy but can be transformed into larger oligomers structure upon mannose binding (Benito *et al.*, 2007).

In fact, these enzymes have different structure or isoforms at different optimum pH but this different structure of enzyme were not reported to have specific function. It was only stated to have function as to control the entry of sucrose into different utilization (Alegre *et al*., 2009). According to Sturm in year 1999 as stated by Alegre *et al*. in year 2009, for acidic forms of invertase appear to have cell wall or vacuolar localization and structurally related to yeast and bacterial invertase. However, in neutral and alkaline forms were found in the cytosol (Vargas *et al*., 2003).

Further review lead to increase in understanding of invertase function. It is a member of GH32 family of glycoside hydrolases, which include more than 370 enzymes of vegetable and microbial origin. (Guimaraes *et al.*, 2007).It is function to hydrolyse the 1, 4-glycosidic bonds of sucrose and eventually formed equimolar mixtures of glucose and fructose that referred as invert sugar (Marquez *et al.*, 2008). It is a type of enzyme which is used for the inversions of sucrose in the preparation of invert sugar and high fructose syrup (Uma *et al.*, 2010).

Since it is naturally exist, therefore many researchers has been attracted to conduct a study on the contribution from this enzyme to the world nowadays. The Figure 2.2 represents the chemical structure of sucrose hydrolysis reaction catalyzed by invertase.

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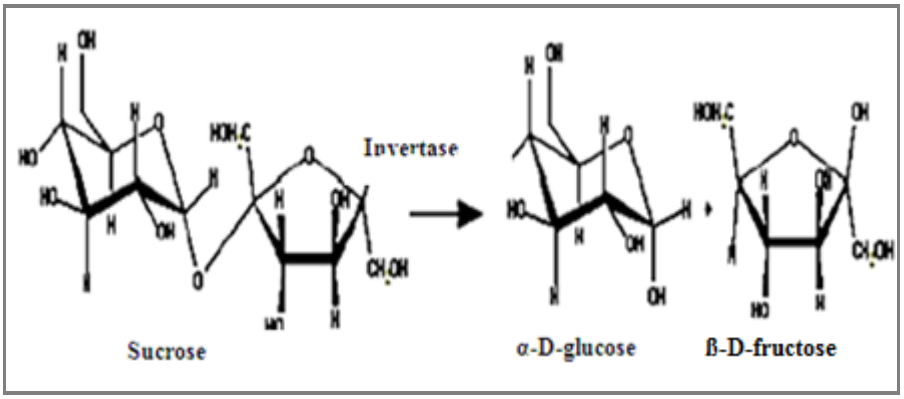

Figure 2.2: An illustration of chemical sucrose hydrolysis reaction catalysed by invertase

Source:**http://www.ensymm.com**. (Retrieved at 3 Nov., 2011)

Nowadays, this enzyme is widely used in confectionery industries and food industries. It is due to the characteristic that produce the fructose compound that have higher sweetening capacity, thus making this enzyme suitable for biotechnological applications, such as the production non-crystallizable sugars and soft centered chocolates (Rubio *et al.*,2003). Other than that, it has been used in the production of non-crystalling creams, jams, artificial honey and in confectionery industry (Emregul *et al.*, 2006).It is also used to produce Fructooligosaccharides (FOS) at higher concentrations of sucrose (Rustiguel *et al.*, 2010). Furthermore, it is also used for diebetics and potentiates iron absorption in children (Gill *et al.*, 2006). Therefore, it is concluded that this study will be contribute large potential in market.

2.3 The Microorganism use to Produce Invertase

This particular enzyme are produced from various type of microorganisms includes fungi, yeast and bacteria. The most type of microorganism that can produced this enzyme is fungi type such as *Aspergillus niger* (Ashokumar *et al.*, 2001), *Aspergillus niveus* (Guimaraes *et al*., 2009), *Aspergillus flavus* (Uma *et al.*, 2010), *Aspergillus phoenicis* (Rustiguel *et al.*, 2010), *Aspergillus fumigatus* (Uma *et al.*, 2010) and etc, and followed by yeasts such as *Saccharomyces cerevisae* (Mona, Nooman, 2009) and bacteria such as *Bacillus macerans* (Samia, 2006). Each type of microorganism requires a specific method to produce invertase enzyme and produced different level of enzyme production.

According to Ashokumar *et al.* in year 2001, by using submerged fermentation of *Aspergillus niger* strain the maximum result is obtained as 18.3 U/L.h for 120 hours fermentation time but the enzyme was optimized by undergo two step to yield 58.3 U/L.h . In addition, this paper also had conducted an investigation on optimization condition of invertase production by using solid state fermentation. As a result, there is existed of invertase productivity which was 81.8 U/L.h with less fermentation time that only required 72 hours yielding optimum productivity compares to submerged fermentation. However, this technique require an additional step compare to submerged fermentation because the sample need to subjected under mechanical squeeze to obtain the extract before the sample undergo the centrifugation step for extracellular enzyme purpose. Therefore, this method becomes undesirable for this research due to the limitation in time.

Other than the Ashokumar *et al.* research paper in year 2001 there are several research papers that used similar *Aspergillus niger.* They are Reddy *et al.* in his research paper in year 2010 and Rubio and Navarro 2006 based on their own method. According to Reddy *et al.* (2010), the investigation of parameters such as pH, temperature and different carbon and nitrogen sources obtain a maximum enzyme activity in 30.84 U/mL. The maximum enzyme activity is obtained at 96 hours fermentation time at pH 3.5 and 30^0C using basal medium that contains 2% molasses as carbon sources supplemented by 0.5 % soya bean meal.

However, it is differed according to Rubio and Navarro (2006) which is use to investigate the effect of raffinose, sucrose and turanose as carbon sources that obtain the maximum 4.0 U/mL enzyme productivity after 48 hours fermentation time. These indicate that the fermentation times are different under different cultivating conditions that influence the invertase production.

Besides the above strain, *Aspergillus niveus* is used to produce extracellular invertase by Guimaraes *et al.* in year 2009 under submerged fermentation. This research paper is conducted to investigate the effect of agroindustrial residues as carbon sources for invertase production. Based on this paper, it is discussed that the presence of sucrose in sugarcane bagasse contribute to the higher enzyme productivity compare to the presence of glucose which is stated that intracellular enzyme is the type of product that become greater compare to extracellular.

Aspergillus flavus is one of the *Aspergillus* strains which able to produce extracellular invertase. According to the Uma *et al.* in year 2010, this strain able to produce invertase and require four days to achieve optimum fermentation cultivating conditions at pH 5.0 and optimum temperature is 30^0C by using 3% inoculum size in Czapek Dox using fruit peel waste as fermentation substrate. This species is culture by using submerged fermentation method that enhance by the addition of sucrose and yeast extract for optimization purpose. Since this research use to purify the invertase therefore the optimum enzyme result is obtained in recovery process as 3.2 % and 5.8 fold.

Further review on the microorganism used to produce this enzyme introduces another *Aspergillus* species strain that able to produce this enzyme. It is *Aspergillus phoenicis* that use as a microorganism in Rustiguel *et al.* fermentation during year 2010. According to this research paper, this type of fungus was grown in Khanna medium that supplemented by wheat bran as carbon sources at temperature 40^0C for 72 hours to obtain the optimum fermentation result. This research paper is similar to the previous reviews which used to purify the extracellular enzyme product to yield 12.5 fold enzymes with 72% recovery. However, the optimum condition that reported is at temperature 60^0C and pH 4.5.

Aspergillus ochraceus is another strain that able to produce this enzyme, according to Guimaraes *et al.* in year 2007, this fungus is able to produce extracellular invertase at maximum yield for 2.68 fold after the purification process. This optimum result is obtained after 96 hours fermentation period at temperature 40^0C by using Khanna medium.

In contrast, the *Bacillus macerans* strain is also able to be used as a fermentation microorganism to produce this enzyme but it is undergo a more complicated fermentation process. It is used by Samia in year 2008 using repeated batch fermentation method. This research had used immobilized *Bacillus macerans* cells in calcium alginate and used for the production of invertase. The purpose of her research is to investigate the influence of alginate concentration, cation concentration, cell to alginate ratio, initial cell loading, curing time and bead diameter on conversion of sucrose to inverted syrup on fermentation product. She had used the immobilized cells in shake flasks study to consider the optimum parameter of her investigation. Finally, this researcher had proposed that the optimum parameter gained from the study to be 3% (w/v) sodium alginate, 3% (w/v) calcium chloride with 2 hours curing time, 200 alginate beads per flask with 2 mm bead diameter. This optimum parameter is based on fermentation of immobilized cells of *Bacillus macerans* in alginate beads that is suggested as more efficient for the production of invertase and can be reused for seven cycles (336 hours) without any loss in their activity and 12 cycles with 72% residual activity.

Other than that, *Saccharomyses Cerevisae NRRL Y-13632* is another microorganism that able to produce invertase. This is reported by Mona and Nooman in year 2009 in their research paper. Basically, this paper is discussed the cultivating condition under solid state fermentation for invertase production using food processing waste. The highest productivity of this research is 272.5 U/g for dry substrate used which is red carrot residues that is experimentally designed with seven nutrients component that contain (g %): $(NH_4)_2SO_4$, 4.5, KH_2PO_4,2.3, $FeSO_4$, 0.01, $MgSO_4.7H_2O$, 0.7, sucrose 5.0; urea,1.1; yeast extract 0.5 (pH 5.0) as described by Ashokumar *et al.* (2001). By undergo four days fermentation time the enzyme yields 29 fold as the optimum value. Besides, this research paper is introduced the pH range that

used to conduct invertase fermentation which is in range 5.0 until 7.0 with the optimum pH reach at pH 6.0 and temperature is 50^0C.

As conclusion, the *Aspergillus niger* strain is the type of species that is desirable for this submerged fermentation study due to the high productivity by using molasses that contains sucrose as the carbon sources which is similar to this research study. As compared to the other strain this species is normally produce higher amount of invertase value in the review of research paper and eventually consider as the most potential strain to be used as to investigate the optimum invertase production in submerged culture during this study.

All of those research papers are summarized in a Table 2.1 as follows for easier comparison purpose:

Table 2.1: Summarization of invertase production by using different microorganism

Species	References	Production
Aspergillus niger	Balasubramaniem *et al.* (2001)	The optimum result is 18.3 U/L.h for 120 h fermentation time but the enzyme was optimized by undergo two steps to yield 58.3 U/L.h.
	Reddy *et al.* (2010)	The maximum 4.0 U/mL of enzyme productivity yields after 48 h fermentation time.
	Rubio and Navarro (2006)	The maximum 4.0 U/mL enzyme productivity after 48 h fermentation time.
Aspergillus niveus	Guimaraes *et al.* (2009)	The optimum enzyme activity in amount of 30.84 ± 0.447 U/mL.
Aspergillus flavus	Uma *et al.* (2010)	The obtained result is 40.41 of total Unit of enzyme founded.